IMMUNITY STUDIES ON ANTHRAX SERUM

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INTRODUCTION

Beginning with the classical work of Pasteur (9)[1] in 1881, wherein he conclusively showed that it was possible through the use of attenuated cultures of *Bacillus anthracis* to immunize animals against otherwise fatal doses of anthrax organisms, various investigators have from time to time taken up work along this line and through extensive experimentation have contributed much toward our knowledge of anthrax, its treatment, and methods of immunization against it.

While in general Pasteur's vaccine proved highly satisfactory and has been extensively used with excellent results, it has a number of disadvantages—that is, it requires two handlings of the animals; the desired degree of immunity is not reached until approximately a week or 10 days after the injection of the second vaccine; there is a small percentage of losses in vaccinated animals due directly to the vaccine; and the keeping qualities of the vaccine under unfavorable conditions are not the best (4). These factors led a number of investigators to attempt various modifications of Pasteur's method. It being possible to immunize susceptible animals against anthrax, some workers directed their efforts toward the preparation of an immune serum through hyperimmunization. Thus, in 1895 Marchoux (8), by immunizing sheep according to Pasteur's method and then hyperimmunizing them through injections of increasing doses of virulent anthrax culture, succeeded in preparing a serum capable of producing a passive immunity in susceptible animals. Further work along this line was conducted by Sclavo, Sobernheim, Mendez, Detre, Carini, and Ascoli. These investigators, however, employed larger animals, especially horses, and succeeded in producing potent immune serums. Serum of this type was found to be of considerable value in cases of threatening infection or where anthrax had already made its appearance in a herd, and in the treatment of the disease. The immunity conferred by anthrax serum, however, is of short duration, lasting only a few weeks. To produce a more lasting immunity Sobernheim (10) recommended a simultaneous treatment with serum and vaccine, the vaccine corresponding to Pasteur's second vaccine. Eichhorn (4) obtained good results from the use of serum and spore vaccine and found the method to possess advantages over the Pasteur method. Numerous experiments undertaken

[1] Reference is made by number to "Literature cited," p. 56.

Journal of Agricultural Research,
Washington, D. C.
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Vol. VIII, No. 2
Jan. 8, 1917
Key No. A—27

demonstrated the value of anthrax serum as a curative agent and as a prophylactic when employed simultaneously with anthrax spore vaccine.

Recalling the work on the separation of diphtheria antitoxin by fractioning the serum through the use of ammonium sulphate, the writers undertook the application of this method to anthrax serum and succeeded in producing the antibodies in a concentrated form. Chemical analyses of the serum and globulin preparations were made, and the changes in serum proteins during the course of hyperimmunization of animals against anthrax were studied.

SEPARATION OF PSEUDOGLOBULIN FROM IMMUNE SERUM

The method used was essentially similar to that described by Banzhaf (2) except that serum instead of citrated plasma was used. In all cases the serum was obtained from natural, spontaneously coagulated blood of two horses, which are here designated as horse 48 and horse 96. These had been hyperimmunized by the senior author, using an improved technic described in a previous publication (4). During the course of 6 months a total of 14 preparations of pseudoglobulin were made from 4 lots of serum from each horse. Serum 48 was known to have a high and serum 96 a comparatively low potency.

In the beginning it was not known whether the antibodies in the anthrax serum would withstand heating to 60° C., as in the diphtheria antitoxin preparation. For this reason each lot of serum was divided into two equal parts, one of which was given the heat treatment. Otherwise, the method of fractioning with ammonium sulphate, filtration, and dialysis was the same for both. The volume of serum used in each preparation varied from 600 to 1,200 c. c. (see Table I). Four preparations were simultaneously made—two from serum 48 (heated and not heated) and two from serum 96 (heated and not heated). This was done in order that the results of the subsequent inoculation experiments might be comparable. The serums from the two horses were not mixed, but were used separately in the pseudoglobulin preparation.

The serum was diluted with one-half its volume of water, and saturated ammonium-sulphate solution was added up to 30 per cent saturation—that is, 30 per cent of the total volume of the mixture. Thus, to 1 liter of serum there were added 500 c. c. of water and 643 c. c. of saturated ammonium-sulphate solution. Thirty per cent of the total volume, 2,143 c. c., consisted of saturated ammonium-sulphate solution, 643 c. c. At this concentration euglobulin was precipitated.[1] The mixtures to be heated were placed in an electrically heated drying oven maintained at 60° C. They were contained in 2-liter Erlenmeyer flasks provided with rubber stoppers. With few exceptions they remained in the oven for six hours and were then filtered along with the corresponding mixtures

[1] In diphtheria antitoxin preparation the euglobulin carries down little, if any, of the antitoxin. Banzhaf (2, p. 115).

that had not been heated. Hard papers were used (S. & S. 575, 24 cm. diameter) in glass funnels. The filtration was fairly rapid, not troublesome, and was allowed to go on overnight. The precipitated euglobulin was rejected. The filtrate contained the anthrax-immune bodies, pseudoglobulin and albumin.

The ammonium-sulphate content of the filtrates was now raised to 50 per cent saturation—that is, sufficient was added so that one-half of the final volume of the mixture consisted of saturated ammonium-sulphate solution. Thus, 1 liter of euglobulin filtrate contained 300 c. c. of saturated ammonium-sulphate solution, 233 c. c. of water, and 466 c. c. of serum. The addition of 400 c. c. of the saturated solution resulted in 1,400 c. c. of a mixture containing 700 c. c. of saturated ammonium-sulphate solutions, 233 c. c. of water, and 466 c. c. of serum. Such a mixture is "50 per cent" saturated. At this concentration pseudoglobulin was precipitated, carrying with it the anthrax-immune bodies. The mixtures were filtered as before, on hard papers. The filtrates containing albumin were rejected.

The precipitated pseudoglobulin was then freed from most of the adherent liquid by pressure between filter papers and towels, the precipitate being retained in the filter paper opened to form a semicircle. A convenient arrangement was the following: A towel; on top of this a large ordinary filter paper or two; on this, two filter papers containing precipitates. These two were covered with two ordinary filter papers and a towel, and so on. The pressure at first must be very slight and the filter papers and towels frequently renewed. After two or three days of pressure with gradually increased weights the precipitates were transferred to a press which pressed them into a condition resembling soft cheese. The color was white or nearly white. The pressed precipitates were easily removed from the papers with a spatula, were weighed, and transferred to parchment dialyzing bags. The weights varied, of course, with the quantity of serum used and other factors. From a liter of serum about 125 gm. of moist, pressed pseudoglobulin were obtained. From 1 to 3 c. c. of chloroform were added, depending upon the amount of precipitate, and the bag was tied. The pseudoglobulin was dialyzed for 3 to 4 days against running tap water until only small amounts of sulphate were present in the tap water. The under surface of the bags was submersed in the tap water to a depth of 1 or 2 cm.

At the end of the dialyzing period the bags were opened and the volume of the globulin concentrate was measured. In all cases the reaction to litmus-paper strips was either neutral or faintly amphoteric; the odor of chloroform had disappeared, showing that the chloroform had dialyzed out and that there is no great danger of using too much chloroform in the beginning. The concentrates were odorless or nearly so. They were transferred to glass bottles and kept in a refrigerator. As a preservative 0.5 per cent chloroform was added. This has been found to be a most

suitable serum preservative by Voegtlin (11). The following quotation from his work (p. 118) is of interest:

> From the experiments described in this bulletin it would seem that chloroform when added to serum even to the point of saturation, is not capable of imparting to the serum a degree of toxicity which could be compared with that obtained on mixing serum with phenol and trikresol (0.25 and 0.5 per cent.). Practically the only effect which could be produced by the subdural injection of chloroform serum is the result of an increase in intracranial tension. With the use of the gravity method such results are not very apt to follow and it seems very doubtful that they occur at all. We, therefore, strongly suggest that chloroform be used as a preservative for antimeningitis serum. It is well recognized that serum preserved with chloroform after long standing will show a cloudiness which is probably due to the partial precipitation of the serum proteins. This fact, however, does not alter the efficiency of such a serum in the treatment of the disease.

With one or two exceptions the globulin concentrates were not Berkefeld-filtered. For analytic data on the serums and their concentrates see Table V.

Table I contains the data on the preparations of globulin concentrates from the serum. The figures in the last column are of particular interest. They show that on the average the concentrates from serum 48 contained 46 per cent, and from serum 96, 68 per cent, of the original total coagulable protein in the serum used in the preparations. In the inoculation tests serum 48 and globulin 48 were generally more potent than serum 96 and globulin 96.

TABLE I.—*Data on the preparations of globulin concentrates from serum*

Date when blood was drawn.	Volume of serum used for globulin concentration.	Heated at 60° C.	Resultant volume of globulin concentrate.	Total coagulable protein in serum used.	Total coagulable protein in concentrate.	Total protein in concentrate.	
	SERUM 48						
	C. c.	*Hours.*	*C. c.*	*Gm.*	*Gm.*	*Per cent.*	
July 26, 1915	1,000	(a)	220	81.7	38.6	47	
Sept. 21, 1915	650	0	190	49.8	27.0	54	
Do	650	6	115	49.8	15.7	32	
Nov. 5, 1915	900	0	220	61.2	33.4	55	Average, 46
Do	900	6	240	61.2	33.6	55	
Jan. 6, 1916	700	0	92	47.2	19.4	41	
Do	700	6	95	47.2	20.0	42	
	SERUM 96						
July 26, 1915	1,200	(a)	460	97.8	78.3	80	
Sept. 21, 1915	650	0	255	55.9	36.8	66	
Do	650	3	235	55.9	33.2	59	
Nov. 5, 1915	900	0	404	79.8	59.5	74	Average, 68
Do	900	6	400	79.8	60.1	75	
Jan. 6, 1916	700	0	146	55.2	32.8	59	
Do	700	6	158	55.2	34.6	63	

[a] Heated in water bath at 60° C. until temperature inside of flasks was very near 60°.

Ascoli (1) separated the pseudoglobulin from anthrax serum and showed that the immune bodies were contained in this fraction. Apparently the technic of serum fractioning was not sufficiently developed at that time to enable Ascoli to concentrate the pseudoglobulin into a small volume as well as to separate it from the serum.

In his very extensive monograph on anthrax, Sobernheim (10) makes no mention of the fractionation of the immune serum, probably because the serum itself was satisfactory for most purposes (p. 696).

ANIMAL-INOCULATION TESTS

The protective power of the globulin concentrates was determined by inoculation experiments, mostly on guinea pigs, of which 263 were used. The preliminary experiments soon showed that the immune bodies were present in the globulin preparations. It is highly probable that the loss of immune bodies during the concentration was not very great; but an exact statement is not possible because at the present time there is neither a unit of anthrax toxin nor of immune body known. They have not yet been studied sufficiently to be standardized. The general statement may be made that the globulin concentrates are more potent than an equal volume of the corresponding serum. Only those details of the tests which are of special interest are mentioned. One of the main objects of the tests was to obtain data that might throw light on the problem of the nature of the immune bodies—whether they were different from or identical with the pseudoglobulin. The tests made so far are not easy to interpret, and little light is thrown by them on the problem.

In test 5, Table II, it was desired to ascertain whether a given weight of pseudoglobulin had the same protective power when present alone as in the globulin preparations, and when present with all the other serum constituents as in the serum administered.

In the first animal-inoculation tests the virus employed was a 24-hour bouillon culture of an attenuated strain of *Bacillus anthracis*, prepared by inoculating from an agar culture the amount of anthrax bacilli that can be taken up on a standard loop into a tube containing 10 c. c. of bouillon; 0.25 c. c. of such a culture constituted the dose. Later, however, a standardized suspension of anthrax spores in normal salt solution (4,000,000 spores per cubic centimeter) was employed, the dose also being 0.25 c. c. The injection of the serum and globulin preparations was made intraperitoneally, followed in 48 hours by a subcutaneous injection of the virus. Virus "Davis-C" represents a culture of *B. anthracis* uniformly fatal for guinea pigs and rabbits; "Davis-D" is fatal for guinea pigs but not rabbits; and "Chestertown" is fatal for sheep, cattle, and horses.

From the results obtained (Table II) on 3 lóts of 12 guinea pigs, which had been injected with varying amounts of serum 48 and globulin 48, it would seem that a given weight of globulin had approximately

the same protective power whether present alone or in the serum. But the results obtained at the same time with serum 96 and globulin 96 do not substantiate this view. It is to be noticed that three guinea pigs that received 1 c. c. each of globulin 96, containing 0.147 gm. of pseudoglobulin, died before the close of the test, as well as three that received 1 c. c. each of the heated globulin 96, containing 0.150 gm. of pseudoglobulin. On the other hand, two guinea pigs out of three that had received 0.075 gm. of globulin (mostly pseudoglobulin, with a small amount of euglobulin) in the form of 1 c. c. of serum 96 survived. Likewise, two out of three survived after receiving 2 c. c. of serum 96. From these latter results alone the inference might be drawn that a protective action existed in the serum which was absent in the globulin preparations. The early death of practically all the guinea pigs that received globulin 96 (Table II) led to the suspicion that the virus C was too strong.[1] Accordingly test 5 was repeated, using the weaker virus D and increasing the quantities of globulin 96 injected (test 7, Table III); otherwise the two tests were the same. (For a description of the virus, technic of the injections, etc., see page 41.)

In test 7 (Table III) the results obtained with globulin 48 are practically the same as those obtained in test 5 (Table II). A larger number of guinea pigs did not survive, although a weaker virus was used. Plainly serum 48 protected a larger number of guinea pigs against virus D than against virus C. In test 7 the large number of survivals with serum 48 as compared with globulin 48 would indicate that the serum contained protective bodies which were absent in the globulin preparations. This is just the reverse of the result in test 5, in which the stronger virus was used. For serum 96 and globulin 96, the results in test 7 are essentially similar to those obtained in test 5, except that a few more of the globulin guinea pigs survived. It is apparent from the results that no definite statements can be made as to whether the survivals were due to the weaker virus or the increased amounts of globulin.

In the tests that were made the protective power of globulin 48 heated was as great as that of globulin 48 unheated, although the former was prepared from serum heated for six hours in a 60° C. air oven in the presence of one-third saturation ammonium sulphate. In so far as the flasks containing the mixtures to be heated were at room temperature when placed in the oven, it is practically certain that the temperature of 60° C. was actually reached inside the flasks only toward the end of the heating period. This method of heating was first used because it was desired to heat the mixture up to 60° C. without exposing any part of it to a temperature much higher than this. In general it was similar to the method of heating described by Banzhaf (2, p. 115). On the other hand, globulin 96, heated, was not as potent in protective power as the corre-

[1] When the tests were in progress the time of death of the animals was noted almost every hour, day and night. Little would be gained by inserting these figures into the tables.

sponding unheated preparation. Just why heat should be detrimental to one serum and apparently without effect on the other is difficult to say. It is possible that serum 48, being the more potent of the two, lost only a relatively small part of its total potency when heated; while serum 96, being comparatively weak, lost a relatively large part of its potency under the same treatment. At the present state of our knowledge there seems to be no need for the heat treatment, although future work may indicate its desirability.

TABLE II.—*Results of test 5 at 72 and 144 hours after the inoculation of guinea pigs with virus C. Seventy-two guinea pigs were inoculated with serum and globulin on Dec. 11, 1915, and with 0.25 c. c. of virus C on Dec. 13, 1915. Six additional guinea pigs (controls) received only virus* [a]

Serum 48.				Globulin 48.							
		Result.		Not heated.				Heated.			
Dose.	Globulin in dose.	72 hours.	144 hours.	Dose.	Globulin in dose.	Result. 72 hours.	Result. 144 hours.	Dose.	Globulin in dose.	Result. 72 hours.	Result. 144 hours.
C. c.	*Gm.*	Died...	.....	*C. c.*	*Gm.*	Died...	.....	*C. c.*	*Gm.*	Died...	.....
1	0.044	Lived..	Lived.	0.25	0.038	Lived..	Died.	0.25	0.035	Lived..	Died.
		..do...	Do.			..do...	Do.			..do...	Do.
		Died...	.....			..do...	Died.			Died...	.....
2	.088	Lived..	Died.	.50	.076	..do...	Lived.	.50	.070	Lived..	Lived.
		..do...	Do.			..do...	Died.			..do...	Do.
		..do...	Do.			..do...	Lived.			..do...	Died.
3	.131	..do...	Do.	.75	.114	..do...	Do.	.75	.105	..do...	Do.
		..do...	Do.			..do...	Do.			..do...	Lived.
		..do...	Do.			Died...	.....			..do...	Died.
4	.175	..do...	Lived.	1.00	.152	Lived..	Died.	1.00	.140	..do...	Do.
		..do...	Do.			..do...	Lived.			..do...	Lived.
Serum 96.				Globulin 96.							
C. c.	*Gm.*	Lived..	Died.	*C. c.*	*Gm.*	Died...	.....	*C. c.*	*Gm.*	Died...	.....
1	0.075	..do...	Lived.	0.25	0.037	do.....	.....	0.25	0.037	..do...	.....
		..do...	Do.			Lived..	Died.			..do...	.....
		..do...	Died.			Died...	.....			..do...	.....
2	.151	..do...	Lived.	.50	.074	..do...	.....	.50	.075	..do...	.....
		..do...	Do.			Lived..	Died.			..do...	.....
		Died...	.....			Died...	.....			..do...	.....
3	.226	Lived..	Lived.	.75	.111	..do...	.....	.75	.112	..do...	.....
		..do...	Do.			..do...	.....			..do...	.....
		..do...	Do.			..do...	.....			..do...	.....
4	.302	..do...	Do.	1.00	.147	..do...	.....	1.00	.150	..do...	.....
		..do...	Do.			..do...	.....			Lived..	Died.

[a] The six control guinea pigs died in less than 72 hours.
Globulins were prepared from blood drawn on November 5, 1915.
In serums 48 and 96 the globulin consisted largely of pseudoglobulin; the globulin preparations contained only pseudoglobulin (see page 39).
In making the heated preparations the serum was heated for six hours at 60° C.

If Tables II and III be divided horizontally by a line running between the 2 and 3 c. c. doses of serum, and the number of survivals in the upper half compared with those in the lower, it will be apparent that the protective action of neither serum nor globulin preparations was strictly proportional to the dose. In Table II there were 10 survivals in the upper

half and 13 in the lower. In Table III there were 18 survivals in the upper and 17 in the lower. These results indicate that the dose is only one of the many factors which decide the course of an individual test. They also indicate the desirability of large numbers of results before generalizations are made.

TABLE III.—*Results of test 7 at 72 and 144 hours after the inoculation of guinea pigs with virus D. Seventy-two guinea pigs were inoculated with serum and globulin on Jan. 15, 1916, and with 0.25 c. c. of virus D on January 17, 1916. Six additional guinea pigs (controls) received only virus* [a]

Serum 48.				Globulin 48.							
		Result.		Not heated.				Heated.			
Dose.	Globulin in dose.	72 hours.	144 hours.	Dose.	Globulin in dose.	Result. 72 hours.	Result. 144 hours.	Dose.	Globulin in dose.	Result. 72 hours.	Result. 144 hours.
C. c.	*Gm.*	Lived.	Died.	*C. c.*	*Gm.*	Died.	.....	*C. c.*	*Gm.*	Lived.	Died.
1	0.044	..do...	Lived.	0.25	0.038	Lived.	Died.	0.25	0.035	..do...	Lived.
		..do...	Do.			..do...	Lived.			..do...	Do.
		..do...	Died.			Died.	.....			Died.	.....
2	.088	..do...	Lived.	.50	.076	Lived.	Died.	.50	.070	Lived.	Died.
		..do...	Do.			..do...	Lived.			..do...	Do.
		..do...	Died.			..do...	Died.			Died.	.....
3	.131	..do...	Lived.	.75	.114	..do...	Do.	.75	.105	Lived.	Lived.
		..do...	Do.			..do...	Lived.			Died.	.....
		..do...	Do.			Died.	.....			Lived.	Died.
4	.175	..do...	Do.	1.00	.152	..do...	.....	1.00	.140	..do...	Lived.
		..do...	Do.			Lived.	Lived.			..do...	Do.

Serum 96.				Globulin 96.							
C. c.	*Gm.*	Died.	.....	*C. c.*	*Gm.*	Lived.	Died.	*C. c.*	*Gm.*	Died.	.....
1	0.075	Lived.	Lived.	0.50	0.074	..do...	Lived.	0.50	0.075	..do...	.....
		..do...	Do.			..do...	Do.			Lived.	Died.
		..do...	Do.			Died.	.....			..do...	Do.
2	.151	..do...	Do.	1.00	.147	..do...	.....	1.00	.150	..do...	Lived.
		..do...	Do.			Lived.	Lived.			..do...	Do.
		Died.	.....			Died.	.....			Died.	.....
3	.226	Lived.	Died.	1.50	.221	Lived.	Lived.	1.50	.225	..do...	.....
		..do...	Lived.			..do...	Do.			Lived.	Lived.
		..do...	Died.			..do...	Died.			Died.	.....
4	.302	..do...	Lived.	2.00	.294	..do...	Do.	2.00	.300	Lived.	Died.
		..do...	Do.			..do...	Do.			..do...	Lived.

[a] Of the 6 control guinea pigs, 3 died in less than 72 hours and the remaining 3 died in less than 144 hours. Globulins prepared from blood drawn Nov. 5, 1915.
In serums 48 and 96 the globulin consisted largely of pseudoglobulin; the globulin preparations contained only pseudoglobulin (see p. 39).
In making the heated preparations the serum was heated for 6 hours at 60° C.

In test 6 the protective action of the globulin preparations on horses, calves, and sheep was demonstrated. The results are contained in Table IV.

All of the other globulin preparations were found to be potent when tested. For the reasons already stated, the protective power of the different preparations can be measured only approximately, and therefore the influence of heat, the relation between globulin contents and potency, etc., will remain in the problematic stage until standardized units of anthrax toxin and antitoxin are available for experimental purposes.

TABLE IV.—*Test 6: Results of inoculations of larger animals. The animals were inoculated with globulin on Dec. 17, 1915, and with virus "Chestertown" on Dec. 21, 1915*[a]

Animal No.	Dose of globulin.	Pseudo-globulin in dose.	Dose of virus.	Result.
		Gm.	*C. c.*	
Horse 132	5 c. c. globulin 48	0.760	0.5	Alive, Jan. 4, 1916.
Horse 130	5 c. c. globulin 48 (heated)	.700	.5	Do.
Horse 143	8 c. c. globulin 96	1.179	.5	Dead, Dec. 30, 1915.
Horse 140	8 c. c. globulin 96 (heated)	1.202	.5	Dead, Dec. 31, 1915.
Horse 133	Control		.5	Dead, Dec. 27, 1915.
Calf 86	5 c. c. globulin 48	.760	.5	Alive, Jan. 4, 1916.
Calf 70	5 c. c. globulin 48 (heated)	.700	.5	Do.
Calf 87	8 c. c. globulin 96	1.179	.5	Do.
Calf 69	8 c. c. globulin 96 (heated)	1.202	.5	Do.
Calf —	Control		.5	Do.
Sheep 507	5 c. c. globulin 48	.760	.25	Dead, Dec. 30, 1915.
Sheep 506	5 c. c. globulin 48 (heated)	.700	.25	Alive, Jan. 4, 1916.
Sheep 505	8 c. c. globulin 96	1.179	.25	Dead, Dec. 23, 1915.
Sheep 510	8 c. c. globulin 96 (heated)	1.202	.25	Do.
Sheep 502	Control		.25	Do.

[a] In making the heated globulin preparations the serum was heated 6 hours at 60° C. The globulins were prepared from blood drawn November 5, 1915.

During a recent outbreak of anthrax in a herd of animals near Richmond, Va., anthrax-globulin preparations were used with very good results. In the course of a week several cows had been lost on this farm and others were sick. An investigation showed the presence of anthrax infection. At this time three cows had high temperatures (104°–106° F.) and were manifesting severe symptoms of the disease. Twenty c. c. of globulin prepared from serum 48 were administered intravenously to each of the three animals. One of the animals was in a dying condition at the time the injection was made and died shortly afterwards. The next morning a decided drop in temperature was noted in the two other animals. Another injection of 20 c. c. of globulin was administered that afternoon. Complete recovery resulted in both cases. Prophylactic treatment was given to 244 head of cattle and 25 horses and mules, consisting of injections of 6 c. c. of globulin where that prepared from serum 96 was used, and 4 c. c. of the serum 48 preparation, administered simultaneously with 1 c. c. of a standardized anthrax-spore vaccine. Up to the present time no additional losses from anthrax have been reported in this herd.

CHEMICAL ANALYSES OF SERUM AND GLOBULIN PREPARATIONS

The first few analyses of serum and globulin were made by the methods described by Banzhaf and Gibson (3). These methods were found to be extremely laborious. If, after precipitating globulin by one-half saturation ammonium sulphate, the mixture was filtered through ordinary ashless paper, the filtrates were cloudy, and a sharp separation of precipitate from filtrate was uncertain. If filtered on hard paper (S. & S. 575), the filtrates were clear, but filtration was extremely slow and evaporation

probably great. It is believed that the use of the centrifuge, as described below, for the purpose of separating the globulin precipitate from the "filtrate" is a marked improvement over the method of filtering. The electrically driven centrifuge used had a revolving head of 12 inches diameter and could carry eight tubes of slightly more than 100 c. c. capacity. To effect a good separation, it must be run for 30 to 40 minutes at about 3,000 revolutions per minute.

In order that the results obtained may be comparable with those of Banzhaf, Gibson, and other investigators, the general method of analysis was similar to that used by them. The results contained in Table V and represented graphically in figure 1 were obtained by the following methods:

TOTAL COAGULABLE PROTEIN.—Ten c. c. of serum are pipetted into a 400 c. c. beaker, 300 c. c. of distilled water added and heated not quite to the boiling point. Two and one-half c. c. of $N/5$ acetic acid (1.2 per cent) are added. This flocculates the proteins at once. On account of the higher protein content of the globulin preparations, less than 10 c. c. may be taken, 5 c. c. being a convenient quantity. Less acetic acid should be used; 0.5 to 1 c. c. will flocculate the protein. After the flocculation has taken place, the solution is brought up to the boiling point for a minute, is allowed to cool, filtered on dry, weighed papers, then washed with small amounts of alcohol and ether, dried to constant weight at 100° C., and weighed. (Further details are given on page 48.) This is a simple, easy determination, and duplicates seldom differ more than 3 or 4 mgm. The filtrates should be water-clear or nearly so.

TOTAL GLOBULIN.—With a pipette 10 c. c. of serum (or globulin preparation or other product) is transferred to a centrifuge tube having a capacity of about 105 c. c. (size of tube 165 by 30 mm.). Forty c. c. of water and 50 c. c. of a saturated ammonium-sulphate solution are then added and centrifuged for 40 minutes at about 3,000 revolutions per minute. If the centrifuge runs smoothly, the precipitated globulin will be firmly packed to the bottom of the tube, leaving the supernatant fluid clear or faintly opalescent. Generally the supernatant fluid was not clear enough; it was then poured off into a second centrifuge tube and run again. On inverting the tube for complete drainage, none of the precipitate should be lost; it packs easily and firmly to the bottom. The volume of the supernatant fluid was noted, and the fluid was then rejected. This was done so that, if desired, corrections could be made for the fluid inclosed in the precipitate. The volume poured off was generally near 85 c. c., and varied between 80 and 90 c. c., according to the amount of precipitate. The corrections were not calculated, as their use at this stage would have been premature. The method can be still further improved before such corrections will be useful.

The globulin precipitates in the bottom of the centrifuge tubes are dissolved by the addition of distilled water, transferred to 400 c. c.

beakers, and heated as before to coagulate the protein. Only a small quantity of acetic acid is necessary for flocculation—usually 0.5 to 1 c. c. of the *N/5* acid was sufficient. The precipitated protein is then filtered on weighed papers, washed till free, or almost free, from sulphate, then washed with small amounts of alcohol and ether, dried, and weighed as in the determination of total coagulable protein.

If desired, the globulin precipitate may be dissolved in 40 c. c. of water and again precipitated by the addition of 50 c. c. of saturated ammonium sulphate. This may free the precipitate of traces of albumin, but the loss of globulin at the same time probably makes this an unnecessary step.

Evaporation from the free surfaces of the fluids in the tubes during a 40 minutes' run in the centrifuge was found to be negligible, amounting to less than 1 or 2 c. c.

Good duplicates are easily obtainable. In 23 determinations the duplicates differed from 1 to 16 mgm., with an average of 7 mgm. This does not include a few determinations that were repeated because the duplicates differed enough to indicate error. Much depends upon the condition of the centrifuge. This must be a high-speed, smooth-running apparatus, which slows down smoothly. Practically the same results are obtained when the same serum is used for two globulin determinations about one month apart. The serums were preserved with 0.5 per cent chloroform (likewise the globulin preparations) and kept in a refrigerator.

The object of precipitating one volume of serum in a final dilution of 10 volumes of one-half saturated ammonium-sulphate solution is to prevent the contamination of the precipitate with albumin, which is said to be absorbed. This is the reason why the precipitate is dissolved and reprecipitated by some workers. While this procedure may be advisable for certain analytic purposes, it is objectionable when such results are to be used in connection with a study of antitoxin or similar products obtained by precipitating one volume of serum in a final dilution of three volumes of one-half ammonium-sulphate solution. This is one of the reasons why the analytic method has been further modified and improved so that the precipitation of globulin in the analyses and in the separation of large quantities of globulin for therapeutic use are both accomplished under the same conditions.

The filter papers used were S. & S. 589 "white ribbon," 15 cm. These were placed in weighing bottles, 50 by 40 cm., dried for six hours in an electrically heated air oven at 100° C., and weighed. They were then dried a second time for two hours and weighed to be certain that the weight was "constant." Almost invariably the second weighings differed from the first by 2 or 3 mgm. Drying the papers to an absolutely constant weight seldom occurred. Two empty weighing bottles were dried along with the others. Their weight, which was taken several times, varied only a fraction of a milligram. All weighings were to the nearest

milligram. The second weight of the paper and bottle was used, regardless of whether it was higher or lower than the first.

After being used for filtration, the papers containing a precipitate were replaced in their respective bottles, dried for 14 hours in the air oven at 100° C., and weighed. They were then dried a second time for two hours and weighed. It was found that 14 hours' drying almost always dried the papers and precipitates completely; the second weighing differed from the first only by 2 or 3 mgm. Occasionally, when the second weighing was less than the first by 6 mgm. or more, a third drying and weighing was made of the particular bottle. The lesser of the two weights was used. As is well known, the increase in weight after prolonged drying is due to oxidation.

A large number of blank filtrations, 23 in all, were made as follows: Through a weighed paper a filtrate obtained from a globulin or similar determination was passed. The paper was washed free from sulphate, then with alcohol and ether, dried 14 hours, and weighed as if it had contained a precipitate. The object was to ascertain the extent of the change in weight due to the mechanical handling, drying, etc. Out of the 23 blanks, the differences were 3 mgm., or less, in 18 blanks; generally there was a slight increase in weight. In the 5 other blanks the differences were 5, 5, 6, 6, and 7 mgm.; 3 of these were gains and 2 were losses in weight. These blanks were not used in correcting the weights of precipitates, as they were small enough to be negligible.

In so far as the weight of a precipitate is obtained by difference, it is obvious that care must be used to be certain that the conditions of drying, etc., are such as to lead to a minimum of error. It is probable that some investigators have not realized that errors of a few milligrams are almost unavoidable; one investigator using this method published results to the tenth of a milligram. It is believed that a minimum of error will result under the following conditions: If the first and second weights of the empty dry papers generally differ by 3 mgm. or less, either weight should be used consistently—that is, either the first weight should be used throughout or the second weight throughout, regardless of whether the differences are plus or minus. After drying the paper and precipitate, the minimal weight should be used, regardless of whether this is the first, second, or even third. Should differences of more than 3 mgm. appear often between consecutive weighings, it is probable that the drying was incomplete.

The analytic data obtained on the serums of horses 48 and 96 and mules 148 and 149 are contained in Table V, together with the data obtained on the globulin preparations. The data in Tables I to IV are obtained from those of Table V. It will be apparent from the last table that when a different technic is used in the determination of globulin in the same serum the results are different. Whenever several results were obtained on one serum that result obtained by single precipitation and centrifuging was regarded as correct. The variations from this

correct result are recorded because they indicate, to some extent, just how the results are affected by the differences in technic. For most purposes it is probable that any method will yield results that are comparable and useful. One of the objects of the repeated trials was to develop a technic by which it would be possible accurately to determine euglobulin, pseudoglobulin, and albumin separately in any one sample of serum, so that the sum of the three determinations would almost exactly equal the total coagulable protein in the same serum. Theoretically this should be true; practically the numerous sources of unavoidable error do not permit such accurate work.

TABLE V.—*Analytic data on serums and globulin preparations*

Date blood was drawn.	In 10 c. c. of—	Total coagulable protein.		Total globulin.[a]	Total globulin in total protein.
		Serum.	Globulin.[a]		
		Gm.	*Gm.*	*Gm.*	*Per cent.*
July 26, 1915	Serum 48	0.817		[b] 0.643	79
Do	Globulin 48		1.757		
Sept. 21, 1915	Serum 48	.767		[c] .503	66
Do	Globulin 48		1.420		
Do	Globulin 48 (heated)		1.365		
Nov. 5, 1915	Serum 48	.680		.438	64
		[d] .672		[d] .457	
Do	Globulin 48		1.520		
Do	Globulin 48 (heated)		1.400		
Jan. 6, 1916	Serum 48	.674		.411	61
Do	Globulin 48		2.110		
Do	Globulin 48 (heated)		2.110		
July 26, 1915	Serum 96	.815		[b] .649	80
Do	Globulin 96		1.704		
Sept. 21, 1915	Serum 96	.861		[c] .669	78
Do	Globulin 96		1.445		
Do	Globulin 96 (heated)		1.415		
Nov. 5, 1915	Serum 96	.887		.754	85
		[d] .893		[d] .744	
Do	Globulin 96		1.474		
Do	Globulin 96 (heated)		1.502		
Jan. 6, 1916	Serum 96	.789		.627	79
Do	Globulin 96		2.247		
Do	Globulin 96 (heated)		2.193		
Aug. 16, 1915	Serum 148	.752		[c] .419	56
Oct. 5, 1915	do	.645		[c] .312	
				[e] .331	
				[f] .333	
				.352	55
Oct. 25, 1915	do	.734		[b] .384	
				.438	60
Nov. 19, 1915	do	.768		.455	59
Dec. 15, 1915	do	.768		.455	59
		[d] .776		[d] .436	
Feb. 2, 1916	do	1.047		.796	76
Mar. 6, 1916	do	.907		.644	71
Aug. 16, 1915	Serum 149	.750		[e] .461	61
				[b] .369	
Oct. 5, 1915	do	.673		[e] .340	
				[e] .337	
				[f] .366	
				.414	62
Oct. 25, 1915	do	.793		[f] .492	
				.533	67
Nov. 19, 1915	do	.745		.494	66
Dec. 15, 1915	do	.730		.414	57

[a] All determinations of globulin are by single precipitation and centrifuging unless otherwise qualified.
[b] Globulin precipitated once and filtered.
[c] Globulin precipitated twice and filtered.
[d] Repetition of previous determination.
[e] Globulin by magnesium-sulphate saturation, twice precipitated and filtered.
[f] Globulin precipitated twice and centrifuged.

In general there was from three to four times as much globulin in the globulin preparations as there was in the same volume of serum. Thus, 10 c. c. of serum 48, January 6, 1916, contained 0.411 gm. of total globulin; about three-fourths of this was pseudoglobulin. The globulin concentrate prepared from this serum contained five times that amount of globulin, 2.110 gm., all of which was pseudoglobulin. This was determined by direct coagulation rather than by precipitation with ammonium sulphate, because the results are more accurate by the former method. Whether the globulin preparations were three or four times as potent in protective power as the same volume of serum can not be stated definitely, for reasons already given. The extent to which other proteins were removed during the globulin concentration is indicated in the last column of Table I.

CHANGES IN THE SERUM PROTEINS DURING THE COURSE OF HYPERIMMUNIZATION

The changes in the serum proteins which take place during the course of hyperimmunization against diphtheria were studied by Hiss and Atkinson (6) and by Ledingham (7). Similar studies were made by Banzhaf and Gibson (3) on the plasma of horses immunized simultaneously against diphtheria and tetanus. They state (3, p. 203):

> The observations of Atkinson and Ledingham, so far as we are aware, are the only determinations of the quantitative relation of the serumglobulin content and antitoxic potency throughout the course of immunization. The subject is of extreme importance because of the constant association of the antisubstance with the serumglobulin.

Hartley's work on this subject (5) was published more recently (see p. 56).

The following quotation from the work of Banzhaf and Gibson (3, p. 206) will serve the purpose of briefly summarizing some of the more important results obtained by the above investigators:

> While the greatest rise in the serumglobulin was usually coincident with maximum antitoxic potency, as already pointed out, the extent of this increase in the serumglobulin was practically independent of the antitoxic potency when the results on more than one horse were contrasted. There may be, then, no relation between the absolute or percentage increase of the serumglobulin and the antitoxic potency in the plasma of different horses. The increase in the serumglobulin of refractory horses may surpass that in the plasma of some of those yielding a high antitoxin.

Obviously a study of anthrax serum during immunization was desirable for at least two reasons: (1) To ascertain whether the increase in potency was accompanied by an increase in globulin, and (2) in so far as the *Bacillus anthracis* does not form a soluble toxin while the bacilli of diphtheria and tetanus do, the serum changes would undoubtedly throw light on both the practical and theoretical knowledge of many related problems in immunity.

In figure 1 the analyses of the serums of horses 48 and 96 and mules 148 and 149 are plotted against the time of bleeding. The immuniza-

tion of the horses was begun in September, 1914, and continued with increasing doses of virus until June, 1915 (4, p. 9). Consequently at the time the analyses were made the serums were past the stage where the characteristic serum changes were to be expected. The immunization of the mules was begun in August, 1915, and mule 149 died on December 23, 1915. It is apparent from figure 1 that the changes in

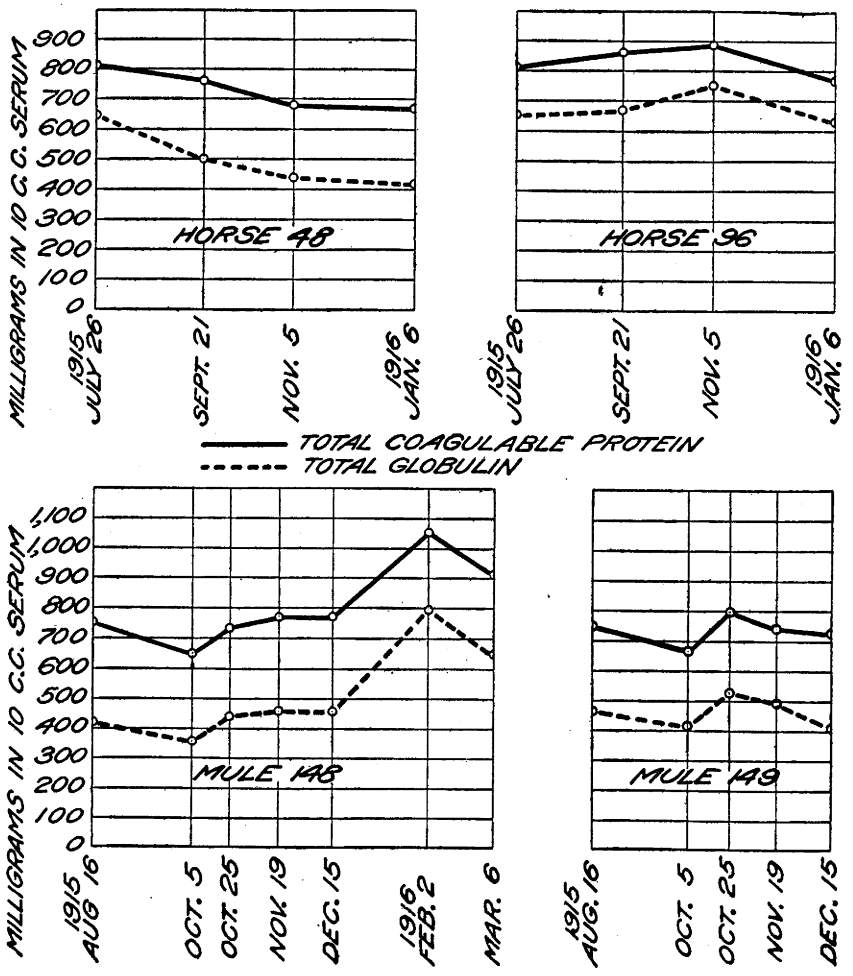

FIG. 1.—Changes in the serum proteins during the course of hyperimmunization.

the serum of mule 149 were similar to those in mule 148 shortly after the immunization was begun. The decline in the condition of mule 149 is believed to be the cause of the downward slope of the curve, or vice versa. The curve for mule 148 is typical in so far as it shows the drop in protein content in the beginning of the immunization, or the "negative phase," followed by a very pronounced rise in protein content. The total coagulable protein and the total globulin rose and fell together.

The term "total globulin" is here used, as it is in Table V, to include the protein precipitated on 50 per cent saturation with ammonium sulphate. In so far as mule 148 received increasing doses of virus during the immunization, the statement may be made that with the rise in total protein and globulin there was a rise in potency; or at least the resistance of the mule was tremendously increased toward anthrax virus.

It would be unsafe to generalize from the curve for a single animal, but obviously in the case of mule 148 the presence of a soluble toxin was not necessary for the production of those serum protein changes usually noticed in diphtheria- and tetanus-immune serums.

Similar changes were observed by Hartley (5, p. 268) in immunization against rinderpest. The increase in total protein and total globulin during immunization has therefore been observed under the following five conditions:

(1) In diphtheria, in which a soluble toxin is involved.

(2) In tetanus, in which a soluble toxin is involved.

(3) In anthrax, in which no soluble toxin is involved according to the present state of our knowledge.

(4) In rinderpest, in which a filterable virus is involved.

(5) In diphtheria, when there is an increase of serum proteins without the production of antibodies.

TREATMENT OF ANTHRAX IN MAN

Since the beginning of the preparation of anthrax serum and globulin by the Bureau of Animal Industry, considerable amounts of these products have been furnished for the treatment of the disease in man, with highly satisfactory results.

Of the serum, the curative dose recommended is 40 c. c. administered subcutaneously in four or five places, repeated as necessary, after intervals of 24 hours, with injections of 25 c. c. In advanced cases the dose may be increased and the injections made intravenously. The dose of the globulin preparation is based on its proportional concentration and is usually from 10 to 15 c. c. The dose being considerably smaller than that of serum, the injection may be safely made intravenously. With other types of protein absent the globulin preparation is superior to the serum in that anaphylactic reaction is minimized.

A number of cases of anthrax in man were treated at Bellevue Hospital, New York, N. Y. Reports from this institution on several of these cases show that a marked lowering of the temperature and reduction of the edematous swelling followed the first injection of serum.

STANDARDIZATION OF ANTHRAX SERUM BY COMPLEMENT FIXATION

It has been found that the serum from various animals treated in an identical manner varies greatly in potency, so that one of the chief difficulties in the production of anthrax serum is its standardization, the various

methods so far employed being quite indefinite and the results variable. The writers have tried several methods of standardization. Sobernheim's method (10), consisting of the intravenous injection of graduated doses of the serum into a series of rabbits, followed immediately with subcutaneous injections of 0.001 loopful of a suspension of virulent anthrax bacilli in saline solution was the first method tried. This, however, did not prove as satisfactory as Ascoli's method. In this test a 24-hour-old attenuated bouillon culture is used, which is of such virulence that when introduced subcutaneously in a 0.25 c. c. dose will kill guinea pigs weighing 350 gm. each in from two to three days.

These cultures must be previously standardized in such a way that they will kill guinea pigs which 24 hours previously have been injected intraperitoneally with 2 c. c. of normal serum. Guinea pigs treated in the same manner and with the same dose of titrated standardized immune blood serum must remain alive. The testing of the serum is carried out on six guinea pigs, each receiving 2 c. c. of the serum intraperitoneally, followed in 24 hours with a subcutaneous injection of the established dose of the test culture. The serum is considered satisfactory for immunization purposes if at least four of the guinea pigs remain alive over six days while the control animals die in three or four days. This test has been modified by using graduated amounts of the serum under test.

The results obtained with the above methods are greatly influenced by the variance in individual susceptibility of the test animals, the character of the virus employed, etc. For this reason the writers have undertaken a series of experiments with the complement-fixation test, with a view to ascertaining its value in the standardization of anthrax serum, and while our work on this phase of the subject is only in the experimental stage and incomplete, the results so far are quite gratifying, and point to the possibility of employing this test as a means of more accurate standardization. So far the serum from two horses and a mule used by the Bureau of Animal Industry in the preparation of anthrax serum, as well as serum from horses in various stages of hyperimmunization obtained from various biological firms of the country, have been employed in this work. At the present time the serums of four horses under the course of hyperimmunization are being studied.

Several antigens have been tried, the best results being obtained with a bouillon culture of a slightly virulent strain of *Bacillus anthracis* (virulent for white mice and occasionally for small guinea pigs). Such a culture is grown at incubator temperature from three to five days and then placed in the refrigerator for two or three weeks or even longer. It is then heated at 60° C. for one-half hour and titrated against a known potent anthrax serum used as a standard.

In standardizing the serum the usual technic employed in the complement-fixation test applies, with the exception that varying quantities of the serum under test are used. So far in the work the writers have

been employing 12 tubes to a test, commencing with 0.2 c. c. of serum in the first tube and graduating the amount down to 0.005 c. c. in the last tube.

In the titration of the antigen and in all standardization tests the results are read at exactly one hour after the addition of the amboceptor and sheep cells, during which time the tubes were kept in the incubator at a temperature of 37.50° C.

Table VI illustrates some of the results obtained, the letters beneath the varying quantities of serum signifying, N, no fixation of complement; S, slight fixation; Gd, good fixation; and Pf, perfect fixation.

TABLE VI.—*Results of complement fixation tests with anthrax serum*

Sample.	Degree of fixation.											
Serum in tubes[a] ..... c. c..	0.2	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.005
A[b]	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.
B[c]	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.
C[d]	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Gd.	S.	N.	N.	N.	N.
D[e]	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Gd.	S.	N.	N.
E[f]	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.
F[g]	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Gd.	S.
G[h]	Pf.	Pf.	Pf.	Pf.	Gd.	Gd.	S.	N.	N.	N.	N.	N.
48[i]	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Gd.	N.	N.
96[j]	Pf.	Pf.	Pf.	Pf.	Gd.	S.	N.	N.	N.	N.	N.	N.
Normal serum[k]	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.

[a] Where the smaller quantities were used, the serum was first diluted to a known strength to avoid inaccuracies likely to occur where minute amounts are employed.
[b] Sample A. Serum from horse which had been vaccinated and subsequently infected with a minute amount of anthrax culture.
[c] Sample B. Same as A.
[d] Sample C. Serum from horse undergoing hyperimmunization. Had received growth from 18 agar slants five-eighths inch surface 17 days previous to the taking of this sample.
[e] Sample D. Serum from horse completely hyperimmunized.
[f] Sample E. Serum from horse which had merely been vaccinated against anthrax.
[g] Sample F. Serum from horse completely hyperimmunized.
[h] Sample G. Serum from hyperimmune horse which had been producing serum for over a year.
[i] Sample 48. Bureau of Animal Industry hyperimmune horse. Serum known to be of good potency through numerous field and laboratory tests.
[j] Sample 96. Bureau of Animal Industry hyperimmune horse. Serum known to be inferior to that from horse 48.
[k] Normal serum. Control.

The above serums were also employed in animal-inoculation tests, in which the results in general corresponded with the complement-fixation test.

While no definite conclusions can be drawn at this time, it appears from the results thus far obtained that as the process of hyperimmunization against anthrax progresses the complement-fixing action of the serum increases. Serum from different animals hyperimmunized by the Bureau of Animal Industry proved to be of different potency, one being of a high potency and the other of a comparatively low potency. (See Table VI.) On applying the complement-fixation test to these samples the complement-fixing value of the serum of highest potency was considerably higher than that of the other serum. Similar results have been obtained with serum from outside sources. A number of other samples of serum from horses in different stages of the hyperimmunization process were tested,

and so far the writers have found that the complement-fixing value has varied with the stage of hyperimmunization—that is, serum from animals in the early stages of the hyperimmunization process possesses little or no complement-fixing value, while that from animals in the advanced stages of the process exhibits considerable complement-fixing properties. The writers are hopeful that further work along this line will bear out the results thus far obtained.

Thus it may prove that by applying a known potent anthrax serum as a standard for the titration of the antigen and for establishing the minimum amount of serum which will cause complete fixation of complement in the presence of the determined amount of antigen, a standard may be established which will permit an accurate standardization of anthrax serum.

No fixation of complement occurs with the serum from animals vaccinated against anthrax or even after they have received the smaller infective doses of virulent culture, a positive reaction occurring only after the beginning of the administration of larger quantities of culture.

SUMMARY

(1) Anthrax serum was fractioned by the methods used in the preparation of diphtheria antitoxin. The anthrax antibodies were associated with the pseudoglobulin fraction.

(2) The globulin preparations contained the antibodies in a concentrated form. This was shown in numerous tests on laboratory animals. The preparations were likewise potent in tests on larger animals—that is, cattle, horses, etc. When administered to human beings (men) infected with anthrax, the globulin preparations were found to have great therapeutic value. However, no data have yet been obtained which permit accurate measurement of the potency of either the serum or the globulin obtained therefrom.

(3) The methods of analysis of serum and similar preparations of globulin have been improved by the use of the centrifuge instead of filtration as a means of separating globulin precipitates from their filtrates. The precipitates are obtained in compact form with a minimal amount of absorbed supernatant fluid. There is no need for reprecipitation.

(4) The changes in the amounts of the serum proteins in a mule undergoing immunization to anthrax were similar to those usually noted in the serum of animals being immunized to diphtheria, tetanus, and rinderpest—that is, there was a pronounced rise in the content of total coagulable protein and total globulin.

(5) Favorable results follow the use of anthrax serum or globulin preparations in the treatment of anthrax in man or animals. The globulin preparation is probably superior to the serum in the treatment of the disease in man, since the dose is smaller, and may be safely given intravenously, and the danger of anaphylaxis is minimized.

(6) The work on the standardization of anthrax serum by complement fixation, while still in an experimental stage and incomplete, points to the possibility of a more accurate means of standardization through its employment.

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